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Antibody profiling of canine IgG responses to the OspC protein of the Lyme disease spirochetes supports a multivalent approach in vaccine and diagnostic assay development

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ABSTRACT

OspC performs essential functions during the enzootic cycle of the Lyme disease (LD) spirochetes. In this study, the specificity of antibody (Ab) responses to OspC was profiled to define the antigenic determinants during infection and after vaccination. Several OspC variants or 'types' were screened with serum from SNAP4Dx C6 positive dogs and with serum from rabbits hyperimmunized with OspC proteins. The OspC type-specific nature of the Ab response revealed that variable domains of OspC are immunodominant during infection and upon vaccination. To assess the potential of OspC to elicit Ab in the context of a bacterin vaccine, OspC production in strains cultivated in vitro was assessed. Immunoblot and indirect immunofluorescent antibody analyses demonstrated that production is low and that only a subset of cells actively produces OspC in vitro, raising questions about the potential of bacterin vaccines to stimulate significant anti-OspC Ab responses. The specificity of the OspC Ab response in experimentally infected mice over time was assessed to determine if domains shielded in the OspC homodimer become accessible and stimulate Ab production as infection progresses. The results demonstrate that the OspC Ab response remains focused on surface exposed variable regions of the protein throughout infection. In contrast to some earlier studies, it is concluded that conserved domains of OspC, including the C7 or C10 domain, do not elicit significant Ab responses during infection or upon vaccination. Collectively, the results indicate that OspC diversity must be considered in vaccine design and in the interpretation of diagnostic assays that employ OspC as a diagnostic antigen.

Introduction

The incidence of Lyme disease (LD) in dogs and human beings is increasing and endemic regions are expanding in many regions of North America and Europe (Mead et al., 2011; Tseng et al., 2015; Sykes and Makiello, 2016). The primary etiological agent of LD in North America is *Borrelia burgdorferi* (Burgdorfer et al., 1982; Benach et al., 1983) while in Europe and Asia, *B. burgdorferi*, *B. garinii*, *B. bavariensis*, and *B. afzelii* are the main species associated with disease (Baranton et al., 1992; Marconi and Garon, 1992; Margos et al., 2013). We refer to this group of bacterial species collectively as the 'LD spirochetes'. LD is transmitted among its diverse mammalian reservoirs and to its incidental hosts (human beings and dogs) by *Ixodes ricinus* complex ticks (Barbour and Hayes, 1986). The number of counties in the USA where *I. scapularis* or *I. pacificus* populations are now established has risen by 49% since 1996 (Eisen et al., 2016). In 2014, the Centers for Disease Control and Prevention reported that the number of LD cases each year in humans is between 300,000 and 450,000 (Hinckley et al., 2014). Data compiled by the Companion Animal Parasitic Council (CAPC) indicate moderate to high risk for LD in dogs in much of the Eastern half of the US and Southern Canada. In 2015, 250,880/4,062,155 (6.2%) tested dogs in the US were antibody (Ab) positive for LD. As detailed by CAPC[1], these numbers represent about 30% of the total, bringing the probable number of Ab positive tests in dogs in 2015 to approximately 800,000.

OspC, an essential virulence factor of the LD spirochetes, has been a major focus of efforts to develop new LD vaccines and diagnostic assays (Grimm et al., 2004; Tilly et al., 2006; Earnhart et al., 2010). OspC production is stimulated in feeding ticks and maintained through early stage infection in mammals, in which it is an immunodominant antigen (Dressler et al., 1993; Fung et al., 1994;

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[1] See: www.capcvet.org/parasite-prevalence-maps.

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Fingerle et al., 1995; Schwan et al., 1995; Gilmore and Piesman, 2000; Pal et al., 2004). The high level expression of OspC in fed ticks and mammals and its antigenicity during early infection are properties that support the use of OspC (or OspC derived proteins) in vaccines and as a diagnostic antigen (Schwan, 2003; Earnhart et al., 2005; Baum et al., 2014). However, OspC is a highly variable protein with ~30 distinct variants or 'types' having been defined (Brisson and Dykhuizen, 2004; Earnhart and Marconi, 2007c; Brisson et al., 2012; Rhodes et al., 2013). OspC types are differentiated by letter designations (e.g. type A, type B).

Different conclusions have been reached regarding the identity of the antigenic determinants of OspC that stimulate Ab responses during infection or upon vaccination with either LD bacterins or recombinant OspC based proteins (Earnhart et al., 2005; Callister et al., 2015). Understanding the antigenic structure of OspC is essential for the translational applications indicated above. Studies in mice and humans suggest that sequence variation, particularly within the coding sequence of the variable loop 5 (L5) and α helix 5 (H5) epitopes of OspC, leads to highly specific Ab responses during infection (Earnhart et al., 2005; Buckles et al., 2006; Earnhart and Marconi, 2007a, 2007b; Baum et al., 2016). Other studies implicate conserved domains of OspC, such as the C7 domain, as immunodominant (Mathiesen et al., 1998b; Gilmore and Mbow, 1999). C7, which has also been referred to as the C10 or pepC10 domain, consists of the C-terminal residues of OspC (Mathiesen et al., 1998a; Wang et al., 1999; Earnhart et al., 2014). In this paper, we use the designation C7/C10 for this domain. It has also been suggested that OspC production patterns may be uniquely different in dogs compared to other mammals (Lovrich et al., 2007; Callister et al., 2015).

In this study, we assessed the specificity and evolution of IgG responses to OspC in the context of natural canine infection, experimental infection in mice and upon hyperimmunization of rabbits with recombinant OspC proteins. In addition, since *B. burgdorferi* cell lysates are the basis for LD bacterin vaccines, we also assessed the production levels of OspC during in vitro cultivation. The results of this study highlight the importance of considering OspC production levels and diversity in vaccine design and diagnostic assay interpretation.

Materials and methods

Bacterial strains, cultivation and serum samples

All strains (Table 1) were cultivated in BSK-H media with 6% rabbit serum (Sigma-Aldrich; 37 °C; 5% CO_2) with 100× antibiotic mix (phosphomycin, rifampicin and amphotericin B; Sigma-Aldrich) as required. Growth was monitored by dark field microscopy. Canine patient serum samples were obtained on a fee for service basis. Information on each dog is presented in Table 2. All dogs were SNAP4Dx (IDEXX) positive for C6 Ab. A subset that was OspC Ab positive, as determined by ELISA (data not shown) using several previously developed OspC epitope based chimeric proteins referred to as chimeritopes (Earnhart and Marconi, 2007b) was selected for further analyses.

Murine studies

Mid-log phase *B. burgdorferi* strains B31MI, LDP56 and LDP84 cells were recovered by centrifugation, washed with phosphate buffered saline (PBS) and 1×10^5 cells were needle inoculated into C3h-HeJ mice ($n = 5$ per group; Jackson Laboratories) (McDowell et al., 2001). Blood was collected from B31MI infected mice at weeks 0, 2, 4, 6 and 12 by tail nick. Blood was collected from LDP56 and LDP84 infected mice at weeks 0 and 4. Mice were euthanazed at either week 4 or 12 and blood was collected by cardiac puncture. Serum was harvested by standard methods. All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals (8th edition) and in accordance with protocols peer reviewed and approved by VCU Institutional Animal Care and Use Committees and/or the Statens Serum Institute.

Generation of OspC recombinant proteins and antiserum

To generate OspC proteins (types A through N), each gene (minus the leader peptide) was PCR amplified (*Pfu* polymerase) from the appropriate isolate (Table 1). The PCR primers (Earnhart et al., 2005, 2010) were designed for ligase independent cloning (LIC) into the pET46Ek-LIC (Novagen). The resulting plasmids were used to generate recombinant proteins in *E. coli* (Miller et al., 2012). The His-tagged proteins were purified using a Ni-Affinity column and an AKTA purification system (GE Healthcare) (Miller et al., 2012). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific). Antiserum to each purified protein was generated in New Zealand white rabbits (Statens Serum Institute) (Theisen, 1996).

Table 1
Borrelia burgdorferi isolates and strains used in this study.

B. burgdorferi isolate or strain	OspC type	Biological source/ description/reference
B31-B31MI	A	A clonal population derived from isolate B31 which was originally obtained from an *Ixodes scapularis* tick (NY, USA). The genome sequence of the B31 parental strain has been sequenced (Fraser et al., 1997).
LPD73	B	Human blood – NY, USA (Earnhart et al., 2005)
LPD84	C	Human blood – NY, USA (Earnhart et al., 2005)
LDP116	D	Human blood – NY-USA (Earnhart et al., 2005)
DRI-03a	H	Tissue biopsy from a purpose bred beagle infected by tick infestation with *Ixodes scapularis* ticks from RI, USA; clonal population (clone a) obtained by sub-surface plating (Rhodes et al., 2013)
B331	I	Human blood, NY, USA
LDP89	K	Human blood, NY, USA (Earnhart et al., 2005)
Veery	M	Veery bird, CT, USA
LDP56	A	Human blood, NY, USA (Earnhart et al., 2005)
DRI40h	F	Tissue biopsy from a purpose bred beagle infected by tick infestation with *Ixodes scapularis* ticks from RI, USA; clonal population (clone h) obtained by sub-surface plating (Rhodes et al., 2013)
LDP74	K	Human blood, NY, USA (Earnhart et al., 2005)
LDP63	N	Human blood, NY, USA (Earnhart et al., 2005)
B31Δ*ospC*	A	*ospC* gene deletion mutant derived from strain B31MI (Earnhart et al., 2010)
B31-HE-OspC	A	Provided by Dr. Patti Rosa (NIH); derived from parental strain B31-5A3; designated in original publication as *ospC*K1/pBSV2G-*ospC* (Tilly et al., 2006); encodes a second copy of the *ospC* gene on plasmid pBSV2G which also encodes gentamicin resistance (gen^R)

Dot blot, SDS–PAGE and immunoblot analyses

Immunoblot strips harboring the purified OspC proteins were generated using the Bio-Dot Apparatus (Bio-Rad). Protein (100 ng; 1× Tris buffered saline, TBS) was added to each well and the proteins were adhered to the nitrocellulose membranes under gravity flow. After washing three times with TBS, the membranes were removed and prepared for immunoblot analysis as described below. All membranes were stained with MemCode Reversible Protein Stain Kit (Thermo Scientific).

For sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), recombinant proteins (500 ng) or *B. burgdorferi* B31MI cell lysate (0.33 optical density units at 600 nm, OD_{600}, per 100 μL SDS–PAGE solution) was boiled, sonicated and fractionated on Any-kD precast Criterion gels (BioRad). One set of gels was stained with Coomassie brilliant blue and others were used to transfer proteins to polyvinylidene fluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (BioRad) and standard methods.

For the immunoblot analyses, nonspecific binding was blocked with 1× PBS containing 0.2% Tween-20 with 5% non-fat dry milk (PBSTM) for 1 h at room temperature (RT). The membranes were probed with canine or rabbit serum (1:500 or 1:15,000 in PBSTM, respectively) and incubated for 1 h at RT. Blots were washed three times with PBST and rabbit anti-dog IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase (1:40,000 in PBSTM; Pierce) was added for 1 h at RT. After washing, bound IgG was detected using the SuperSignal West Pico Western blotting substrate (BioRad).

Indirect immunofluorescence assays

Indirect immunofluorescence assays (IFAs) were conducted as previously described (Earnhart et al., 2010). Cells in PBS were placed on Superfrost Plus slides (Fisher Scientific), air dried, permeabilized with acetone, air dried and then nonspecific binding blocked by the addition of PBSTB (PBST with 3% bovine serum albumin). Slides were screened with mouse pre-immune serum, anti-OspC type A or anti-OspA antiserum (1:25,000 in PBSTB; 30 min), washed with PBST and bound IgG was detected with Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes). The slides were washed, coverslips mounted (ProLong Gold; Molecular Probes), and bound Ab was detected by fluorescence microscopy (BX51; Olympus).

Table 2
Description of canine serum samples and summary of OspC serological analyses.

Dog identification	Breed	State of residence	Recombinant OspC types/scoring of immunoreactivity[a]														Number of OspC types detected by infection serum
			A	M	F	C	D	H	B	I	K	N	E	G	J	L	
PI	Beagle	Unknown[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP2	Labrador retriever	DC	3	2	1	1	1	1	0	0	2	0	1	2	3	0	10
DB	Golden retriever	VA	4	2	3	3	3	3	4	3	3	3	3	3	3	3	14
MT	Yorkshire terrier	OH	1	1	3	0	0	3	0	0	0	0	1	0	0	0	5
BS	Golden retriever	NC	4	1	1	2	1	1	0	0	2	2	3	2	2	1	12
TB	English setter	VA	0	2	2	0	0	0	0	0	0	0	0	1	0	0	3
SW	Golden retriever	VA	1	0	0	0	0	1	0	0	0	0	0	0	0	0	2
PB	Newfoundland	ME	0	0	2	0	0	2	0	0	0	0	0	3	0	0	3
CTR	Beagle	MI	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
GP	Bouvier des Flandres	MD	3	3	2	4	2	2	1	1	0	1	1	2	1	0	12
BM	Mixed breed	MD	2	2	0	2	0	2	0	0	0	2	0	0	0	0	5
LD	Lhasa Apso	CT	0	0	0	0	0	0	0	0	0	0	0	3	0	0	1
TO	Labrador retriever	VA	2	0	0	0	0	0	0	2	0	0	0	0	0	0	2
HS	Beagle	NC	4	4	3	3	4	3	4	4	4	4	4	4	3	4	14
HP	Unknown	Unknown[b]	2	2	0	0	0	0	1	1	2	2	2	3	1	1	10
CTZ	Mixed breed	VA	0	0	0	0	0	3	0	0	2	0	0	0	0	0	2
AH	Mixed breed	PA	2	0	0	0	0	0	0	0	0	0	2	0	0	0	2
SK	Mixed breed	IL	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1
TJ	Great Dane	OH	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2
SM	Retriever	MD	3	0	3	3	3	3	3	2	3	0	2	3	3	3	12
JP	Golden retriever	MD	0	0	0	0	0	3	0	1	0	0	2	4	0	0	4
BH	Mixed breed	VA	3	0	0	0	0	0	0	0	0	2	0	1	0	0	3
AS	Mixed breed	OH	4	0	0	0	0	0	0	0	0	0	0	0	0	0	1
CT	Mixed breed	VA	0	0	0	0	0	0	0	0	0	0	0	3	0	0	1
BH	Border collie	AR	0	0	0	2	0	1	0	3	0	0	0	0	0	0	3
TT	Mixed breed	NY	2	3	0	0	2	2	0	0	0	0	0	3	0	0	5

[a] Reactivity on the dot blots was scored using a scale of 0–4, with 0 indicating no Ab detection and 4 indicating strong Ab detection.
[b] The designation 'unknown' indicates that information about either the breed or state of origin was not available.

Mouse anti-OspA served as a positive control, since OspA is expressed during in vitro cultivation. Pre-immune serum served as a negative control. Cells were also visualized by dark-field microscopy.

Results

Analysis of antibody responses to OspC in naturally infected dogs and in rabbits hyperimmunized with OspC proteins

To assess IgG responses to OspC in infected dogs, serum from 25 C6 Ab positive client owned dogs (SNAP4Dx) was screened against 14 OspC type proteins that represent the major variants found in North America (Earnhart and Marconi, 2007c; Cerar et al., 2016). All dogs were seropositive for one or more OspC variants (Table 2; Fig. 1). Five reacted with a single OspC type, 13 reacted with two to five types and seven reacted with >10 types. The selective recognition of different OspC type proteins indicates that the immunodominant epitopes of OspC must reside within variable domains as opposed to conserved regions, such as the C7/C10 domain.

To assess the specificity of IgG responses to divergent OspC proteins, rabbits were hyperimmunized with OspC type A, B, C, D, H or K protein. Dot blot analyses revealed that immunization elicits OspC type specific Ab (Table 3), supporting the data above, indicating that it is the variable domains of OspC, such as the L5 and/or H5 epitopes, which are immunodominant. These results are also consistent with earlier studies which demonstrated that immunization of mice with a single OspC protein elicits an Ab response with a narrow protective range (Gilmore et al., 1996; Bockenstedt et al., 1997).

Table 3
Specificity of OspC Ab responses in hyperimmunized rabbits.

Serum identification	B	N	C	D	H	K	A
Anti-OspC type B	+	–	–	–	–	–	–
Anti-OspC type C	–	–	++	–	–	++	++
Anti-OspC type D	–	–	–	++	–	–	–
Anti-OspC type H	–	–	–	–	++	–	–
Anti-OspC type K	–	–	–	–	–	++	+
Anti-OspC type A	–	–	–	–	–	–	++
Rabbit pre-immune	–	–	–	–	–	–	–

Analysis of OspC production levels by B. burgdorferi *during in vitro cultivation*

OspC concentration in a bacterin should be proportional to its contribution in eliciting bactericidal Ab responses in vaccinated animals. Since bacterins are produced using in vitro cultivated spirochetes, we assessed OspC production levels in several cultivated strains. SDS–PAGE gels of cell lysates were stained or immunoblotted and screened with anti-OspC type A antiserum (Fig. 2A). Strain B31-HE-OspC served as a positive control (Tilly et al., 2006). Stained gels and immunoblots revealed low or variable levels of OspC production in B31, LDP56 and LDP74 strains (types A and K OspC, respectively). While variable staining of OspC with CBB stain (Alverson et al., 2003) could contribute to the interpretation of these data, we confirmed through staining of SDS–PAGE gels loaded with known amounts of each OspC protein that staining was equal (data not shown). As expected, high levels of OspC were detected in the B31-HE-OspC strain and no OspC was detected in B31Δ*ospC* (Earnhart et al., 2010). Strains LDP84 and DRI40h (OspC types C and F, respectively) (Earnhart et al., 2005; Rhodes et al., 2013) were weakly reactive with the anti-OspC type A antiserum due to both low expression (no visible OspC band upon staining) and weak recognition by anti-type A OspC antiserum. While a visible OspC band was observed in the DRI40h cell lysate upon staining, the protein was only weakly reactive with the anti-OspC type A antiserum. Analysis of OspC expression at the population level using IFAs revealed that only a subset of B31 cells produces OspC during cultivation, whereas all B31-HE-OspC cells produced OspC (Fig. 2B). Immunoblot and IFA analyses of additional *B. burgdorferi* strains also revealed low level, sub-population dependent OspC production (data not shown). In contrast to OspC, OspA, which is known to be produced at high levels in vitro, was produced by all cells.

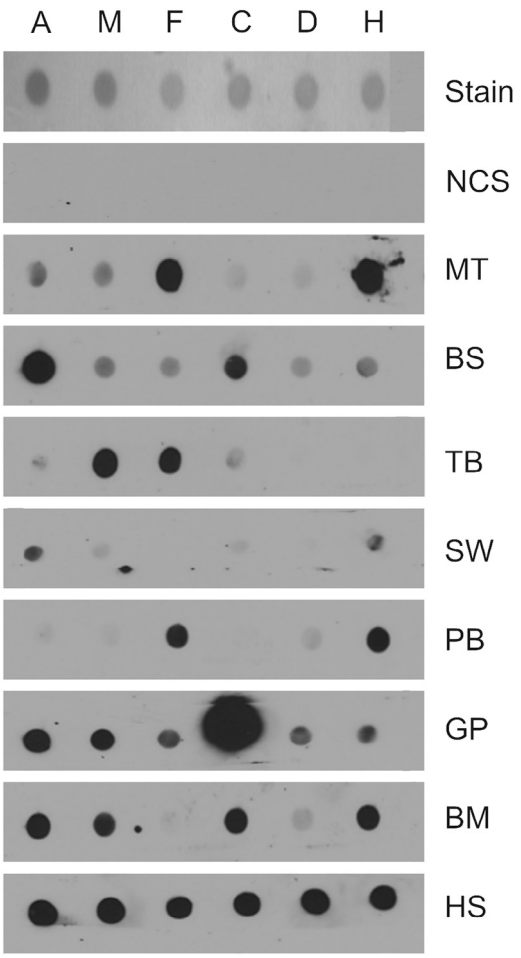

Fig. 1. The IgG response to OspC during natural infection with *Borrelia burgdorferi* in client owned dogs is OspC type specific. Fourteen recombinant OspC proteins were generated, purified and spotted onto nitrocellulose membranes using a dot blotter as detailed in the text. The type identity of each protein is indicated above the top panel (representative data are shown for six recombinant OspC proteins). The dot blot strips were screened with canine serum (indicated by an identification designation, ID) diluted 1:500, as detailed in the methods. Serum from an infection free dog (SNAP4Dx and OspC Ab negative; normal canine serum, NCS) served as a negative control. Twenty-five SNAP4Dx positive dogs were screened (representative data are shown with the complete data set presented in Table 2). To verify similar loading of each protein on the blot, one dot blot was stained with MemCode Reversible Protein Stain Kit (Thermo Scientific). The stained blot is indicated as 'stain'.

Specificity of the anti-OspC antibody response over the course of infection in mice

The evolution of the specificity of the OspC response during infection was assessed by screening OspC proteins with serum harvested at 0, 2, 4, 6 and 12 weeks from mice infected with B31MI (Fig. 3). IgG to type A OspC was detected after week 2, with levels increasing out to week 12. While the Ab response was predominantly directed at OspC type A, and remained so throughout infection, weak cross reactivity with some other OspC types was observed. The Ab response in mice infected with LDP56 at week 4 was also type-specific. In contrast, Ab from mice infected with LDP84 (type C OspC) detected OspC types C, M, A and H. Comparative analysis of the L5 and H5 epitope sequences from OspC types C, M, A and H revealed that segments of the H5 epitope are conserved among OspC types M and C (85% amino acid identity over a 39 amino acid residue stretch), with the sequence differences localized within the N and C-terminal portions of the epitope. These differences would not be expected to interfere with cross reactivity of Ab against the type M and C H5 epitopes. In contrast, amino acid difference scattered throughout the H5 epitopes of OspC types A, C and H (69% identity) would presumably prevent Ab cross reactivity among these OspC types.

Discussion

Analyses of anti-OspC Ab responses in naturally infected dogs using divergent OspC proteins as detection antigens demonstrated significant variation in Ab responses among individual animals. Serum Ab from most infected dogs recognized a limited and variable subset of the most prevalent OspC types of North American LD isolates (Brisson and Dykhuizen, 2004; Earnhart and Marconi, 2007c; Strle et al., 2011). This observation suggests that the dominant immuno-stimulatory determinants of OspC reside within variable domains. Others have postulated that conserved domains, specifically the C7/C10 domain, are immunodominant (Mathiesen et al., 1998a; Bacon et al., 2003; Porwancher et al., 2011; Arnaboldi et al., 2013). However, if that were the case during canine infection, then all OspC variants screened with infection serum would be immunoreactive, regardless of the OspC type identity of the infecting strain(s). Additional evidence arguing against a significant contribution of C7/C10 in OspC Ab responses in canines came from allelic exchange replacement studies (Earnhart et al., 2014). Replacement of the native *ospC* of *B. burgdorferi* B31 with an *ospC* that lacks the coding sequence for the C7/C10 domain had no effect on OspC Ab titers elicited during infection in mice. The weak antigenicity of the C7/C10 domain may indicate that it is not surface exposed on the OspC dimer. OspC atomic structures revealed C7/C10 domain to be structurally unordered and its placement within OspC has not been defined (Eicken et al., 2001; Kumaran et al., 2001).

Type-specific responses to OspC were also observed in rabbits immunized with different OspC type proteins. This observation was surprising, since one might expect buried or masked epitopes hidden in the context of the OspC dimer in viable cells to be accessible in recombinant proteins. The type-specific responses might reflect the highly stable and rigid structure of the OspC homodimer (Eicken et al., 2001; Kumaran et al., 2001). It is noteworthy that the buried interface of the dimer constitutes 22% of the total surface accessible area of each monomer (Kumaran et al., 2001). The data above support the notion that a single OspC protein would be ineffective in stimulating a broadly protective Ab response.

It has been suggested that OspC present in bacterin vaccines may stimulate bactericidal Ab production (LaFleur et al., 2010). However, OspC production during in vitro cultivation has been demonstrated by several research groups to be variable, low level and influenced by temperature (Schwan et al., 1995; Yang et al., 2000; Ohnishi et al., 2001; Ramamoorthy and Scholl-Meeker, 2001; Alverson et al., 2003; Mulay et al., 2009). In this study, IFA and immunoblot analyses were employed to assess OspC production levels at the population level and among strains under standard cultivation conditions (37 °C; BSK-H complete media). Immunoblotting revealed relatively low expression and IFA analyses demonstrated that only a fraction of cells in culture produce OspC. Hence, in the context of a bacterin vaccine, OspC constitutes a minor fraction of the total protein content. This may explain the low level of OspC targeting bactericidal Ab activity detected in the serum of dogs vaccinated with some LD bacterins (Lovrich et al., 2007). LaFleur et al. sought to compensate for this through the use of a two strain bacterin consisting of strain S-1-10 and an *ospAB*$^{-}$ strain (LaFleur et al., 2010). Strains that are unable to produce OspA either as a result of plasmid loss or genetic manipulation tend to produce more OspC than their wild-type counterparts (He et al., 2008; Srivastava and de Silva, 2008). However, it remains to be determined if bacterins,

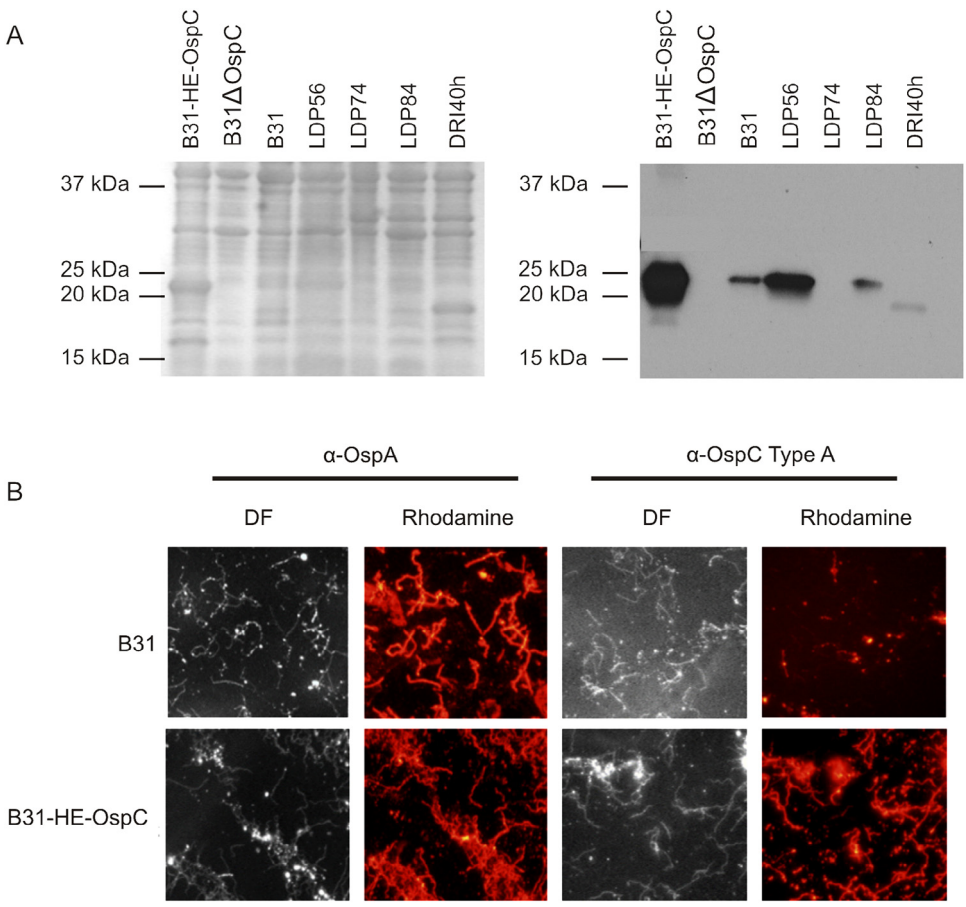

Fig. 2. OspC is expressed at low and variable levels during in vitro cultivation. To assess OspC expression levels during in vitro cultivation, cell lysates of *Borrelia burgdorferi* strains were separated by SDS–PAGE and then stained or immunoblotted. The strains analyzed are indicated above each panel and are described in detail in Table 1. Panel A is an image of a gel stained with Coomassie brilliant blue and panel B is an immunoblot screened with mouse anti (α)-OspC (type A) antiserum with HRP conjugated goat anti-mouse IgG diluted 1:15,000 and 1:40,000, respectively. Molecular weight (MW) standards are indicated. Panel B presents the results of indirect immunofluorescent assay (IFA) analyses of *B. burgdorferi* B31 and *B. burgdorferi* B31-HE-OspC (as labeled). The cells were screened with anti-OspA and anti-OspC type A antiserum, as indicated. The secondary is Alexa Fluor 568-conjugated goat anti-mouse IgG. The cells were visualized by fluorescence microscopy using a rhodamine filter. The corresponding dark field microscopy images are shown.

such as that described above, do in fact harbor elevated amounts of OspC.

To determine if the Ab response to OspC remains type-specific as infection progresses, serum was collected over time from mice infected with B31MI (type A OspC) and used to screen a panel of eight OspC type proteins. Ab specificity remained largely specific for type A OspC, with only minor cross reactivity with other OspC proteins. Hence, even with cell turnover during infection which would liberate OspC from the membrane, conserved portions of the protein do not appear to elicit significant Ab responses.

The specificity of the OspC Ab response has implications for vaccine and diagnostic assay development. Based on the data presented here and in earlier studies (Earnhart and Marconi, 2009; Baum et al., 2014), the broad protective capability of OspC based vaccines is clearly enhanced by the inclusion of multiple epitopes derived from diverse OspC type proteins. Consistent with earlier literature, broad protection cannot be achieved using a single OspC protein or cocktail of OspC proteins (Earnhart and Marconi, 2009). A chimeric OspC epitope protein, referred to as a 'chimeritope' (Earnhart and Marconi, 2007b; Earnhart et al., 2007; Marconi and Earnhart, 2010), has been developed and is a central component of Vanguard crLyme vaccine (Zoetis)[2]. OspC chimeritopes that consist almost entirely of L5 and H5 epitopes from diverse OspC variants have been demonstrated to elicit Ab with bactericidal activity against diverse strains (Earnhart and Marconi, 2007b; Earnhart et al., 2007). Hence, it can be concluded that the C7/C10 domain is not strictly required for stimulating bactericidal Ab. Consistent with this, Lovrich et al. (2007) concluded that, while C7/C10 may stimulate bactericidal Ab in human beings, it does not in dogs.

The data presented here also suggest that a single OspC variant is insufficient to serve as a reliable diagnostic antigen; for example, as shown in Fig. 1, canine TB is clearly seropositive for OspC types F and M. However, canine TB was negative or weakly positive for OspC types A, C, D and H. Hence, the use of an OspC variant other than types F and M as a diagnostic antigen would have resulted in a false negative diagnosis of dog TB. The importance of a multivalent approach can also be gleaned from several published studies that have assessed OspC as a diagnostic antigen. In one study where a single OspC protein (derived from strain 50772) served as a diagnostic antigen, a positive Ab response to OspC was detected in only 3/24 beagles infected by tick infestation, leading the authors to conclude that OspC is unreliable as a diagnostic antigen (Callister et al., 2015). Another study used cell lysates of strain 297 (type E OspC) as the immobilized antigen to assess Ab responses to OspC in infected dogs (Callister et al., 2015). While all dogs assessed in that study were culture positive for the LD spirochetes, only 1/14 was reported to be OspC Ab positive. In light of the fact that OspC

[2] See: www.zoetisus.com/products/dogs/vanguard-crlyme.

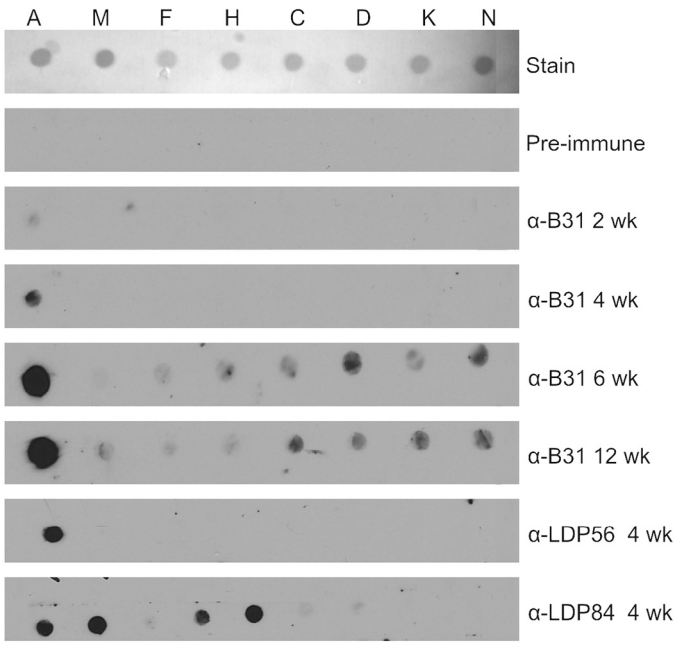

Fig. 3. Analysis of the temporal nature and OspC type specificity of the IgG response in mice infection with a clonal population of *Borrelia burgdorferi* B31 (type A OspC strain). A subset of recombinant OspC proteins was dot blotted and screened with serum collected at different time points from mice infected with *B. burgdorferi* B31. Dot blots were also screened with serum collected at week 4 post-infection with strains LDP56 and LDP84. As a control for loading one membrane was stained with MemCode Reversible Protein Stain Kit (Thermo Scientific). The dot blots were incubated with individual serum from infected dogs and IgG binding detected as detailed above. α, anti.

is required for infection and is a proven early antigen, the lack of detection of an anti-OspC response in the aforementioned studies may instead be due to the use of a single OspC variant as the diagnostic antigen.

Conclusions

The antigenic properties of OspC suggest that careful consideration must be given to OspC diversity in the development of LD vaccines and diagnostic assays. One attractive approach eluded to above to address OspC diversity is the use of OspC chimeritopes, which have the potential to elicit broadly protective Ab responses that can both inhibit spirochete transmission from ticks to mammals and kill spirochetes that enter mammals. The potential exists to extend this technology to development of veterinary and human LD diagnostic antigens as well as in the development of a human LD vaccine.

Conflict of interest statement

This work was supported in part by funds from The Stephen and Alexandra Cohen Foundation[3], Virginia Commonwealth University and Global Lyme Diagnostics. Global Lyme Diagnostics played no role in the study design or in the analysis and interpretation of data.

RT Marconi has a financial interest in canine Lyme disease vaccine (Vanguard crLyme; Zoetis).

[3] See: www.steveandalex.org.

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